

A single bout of resistance exercise triggers mitophagy, potentially involving the ejection of mitochondria in human skeletal muscle

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Abstract

Aim: The present study aimed to investigate the effects of a single bout of resistance exercise on mitophagy in human skeletal muscle (SkM).

Methods: Eight healthy men were recruited to complete an acute bout of one-leg resistance exercise. SkM biopsies were obtained one hour after exercise in the resting leg (Rest-leg) and the contracting leg (Ex-leg). Mitophagy was assessed using protein-related abundance, transmission electron microscopy (TEM), and fluorescence microscopy.

Results: Our results show that acute resistance exercise increased pro-fission protein phosphorylation (DRP1^{Ser616}) and decreased mitophagy markers such as PARKIN and BNIP3L/NIX protein abundance in the Ex-leg. Additionally, mitochondrial complex IV decreased in the Ex-leg when compared to the Rest-leg. In the Ex-leg, TEM and immunofluorescence images showed mitochondrial cristae abnormalities, a mitochondrial fission phenotype, and increased mitophagosome-like structures in both subsarcolemmal and intermyofibrillar mitochondria. We also observed increased mitophagosome-like structures on the subsarcolemmal

cleft and mitochondria in the extracellular space of SkM in the Ex-leg. We stimulated human primary myotubes with CCCP, which mimics mitophagy induction in the Ex-leg, and found that BNIP3L/NIX protein abundance decreased independently of lysosomal degradation. Finally, in another human cohort, we found a negative association between BNIP3L/NIX protein abundance with both mitophagosome-like structures and mitochondrial cristae density in the SkM.

Conclusion: The findings suggest that a single bout of resistance exercise can initiate mitophagy, potentially involving mitochondrial ejection, in human skeletal muscle. BNIP3L/NIX is proposed as a sensitive marker for assessing mitophagy flux in SkM.

KEYWORDS

BNIP3L/NIX, mitochondria cristae, mitochondria dynamics, mitophagy

1 | INTRODUCTION

The human skeletal muscle (SkM), which accounts for approximately 40% of body mass in humans, is a highly active metabolic tissue that plays a crucial role in insulin-stimulated glucose disposal¹ and in locomotion. Mitochondria are essential players in SkM metabolism and function, and their dysfunction or morphology alteration has been associated with lipotoxicity and insulin resistance,^{2,3} as well as sarcopenia and dystrophy in humans.^{4,5} Exercise training is a cornerstone for the improvement of SkM metabolism and the prevention of metabolic diseases.⁶ In particular, resistance exercise provides a potent anabolic stimulus to increase SkM mass and strength.⁷ However, the effects of resistance exercise on mitochondrial function and morphology are not yet fully understood and remain controversial.⁸ Previous research has shown that resistance exercise training increases mitochondrial respiratory capacity in human SkM without increasing citrate synthase activity as a marker of mitochondrial density.^{9–11} Moreover, resistance-trained individuals possess an increased mitochondrial cristae density¹² – a marker of metabolic capacity.^{2,13} Together, these studies suggest that resistance exercise training may enhance the mitochondrial oxidative capacity of SkM, potentially by remodeling the mitochondrial ultrastructure without affecting mitochondrial density. However, the underlying mechanism remains unknown.

Mitochondria are dynamic organelles that adapt their morphology and function in response to intracellular and extracellular stimuli.¹⁴ Their morphology depends on the balance of the two interrelated processes called mitochondrial fusion and mitochondrial fission. Mitochondrial fusion is regulated by mitofusins 1 and 2 (MFN1/2) and optic atrophy 1 protein (OPA1),

connecting outer and inner mitochondrial membranes, respectively.¹⁵ Fusion supports multiple elements of mitochondrial biology such as mtDNA integrity, mitochondrial respiration, and apoptosis. Mitochondrial fission is regulated by dynamin-1-like protein (DRP1), which generates a constricting ring around mitochondria and drives its division.¹⁶ Fission is required to segregate damaged mitochondria for mitophagy¹⁷ – a vital quality control mechanism to preserve a healthy mitochondrial pool¹⁸ that is activated when mitochondria present low membrane potential or irreversible mtDNA damage.^{17,19} So far, two different mechanisms have been described to regulate mitophagy. The first is ubiquitin-dependent and comprises the phosphatase and tensin homolog (PTEN)-induced putative kinase protein 1 (PINK1) – Parkin pathway. The second is ubiquitin-independent, or receptor-mediated, and comprises the proteins BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3), BNIP3-like (BNIP3L/NIX),²⁰ and FUN14 domain-containing 1 (FUNDC1).^{21,22} Both generate an “eat me” signal to engulf the damaged mitochondria into a mitophagosome. The mitophagosome then fuses with the lysosome, resulting in the degradation of the mitochondria into a mitolysosome. However, multiple mitochondrial quality control pathways have been described. A recent study found that damaged mitochondria can be ejected from cardiomyocytes via extracellular vesicles (EVs).²³ A decrease in the mitophagy process induces an accumulation of damaged mitochondria in SkM and SkM deterioration in mice.²⁴ As such, mitophagy could be considered an indispensable mechanism for quality control that could affect mitochondrial oxidative capacity adaptations following resistance exercise in human SkM.

In humans, 8 weeks of endurance training increased the abundance of mitophagy proteins, suggesting an

enhanced capacity for mitophagy.²⁵ A study conducted on rats showed that a single bout of resistance exercise increases in p-DRP1^{Ser616}, an important step in the initiation of mitophagy, suggesting an increase in mitochondria fission. In humans, a single bout of resistance exercise resulted in mitochondrial morphology changes and showed signs of cristae damage in SkM,¹² which could initiate the mitophagy process. However, it remains unknown if resistance exercise can trigger mitophagy in human SkM. Therefore, the aim of the present study was to investigate the impact of a single bout of resistance exercise on mitophagy. We hypothesized that an acute resistance exercise could enhance mitochondrial quality control by inducing fission and mitophagy, which would facilitate the removal of damaged mitochondria in human skeletal muscle.

2 | RESULTS

2.1 | Human study 1

2.1.1 | Acute resistance exercise increases p-DRP1^{Ser616} and decreases PARKIN, BNIP3L/NIX, and mitochondrial complex IV protein abundance in human SkM

After a single bout of resistance exercise, Ex-leg showed increased p-DRP1^{Ser616}, decreased MFN2, and unchanged OPA1 protein abundance compared to the Rest-leg (Figure 1A,B). Autophagy markers LC3I, LC3II, p62, and p-AMPK^{Thr172} protein did not change, but p-mTOR^{Ser2448}, a master regulator of the autophagy pathway,²⁶ increased in the Ex-leg (Figure 1A,C). Mitophagy markers PINK1, FUNDC1, and BNIP3 remained unaltered, while PARKIN and BNIP3L/NIX protein abundance decreased in the Ex-leg (Figure 1A,D). Among OXPHOS protein abundance, complex (C) IV and CIII (trend) decreased in Ex-leg versus Rest-leg (Figure 1A,E). Regarding SkM fiber oxidative capacity, SDH activity (complex II) did not change in Ex-leg (Figure 1F,G).

2.1.2 | Resistance exercise elicits abnormal mitochondrial cristae and mitophagosome-like structures in human SkM

We evaluated mitochondrial morphology through TEM images in intermyofibrillar (IMF) and subsarcolemmal (SSM) mitochondria subpopulations (representative images in Figure 2A). Mitochondrial size did not change in both IMF and SSM subpopulations (Figure 2C,D). However,

mitochondrial circularity increased in Ex-leg in both mitochondrial subpopulations. Abnormal mitochondrial cristae (Figure 2B–D; representative image in Figure 2B) were increased in both IMF and SSM mitochondria, accompanied by an increased fraction of mitophagosome-like structure in Ex-leg (Figure 2B–D; representative image in Figure 2B). Additionally, mitophagosome-like structures on subsarcolemmal clefts were increased in Ex-leg compared to Rest-leg (Figure 2E). Mitophagosome-like structures were detected outside of the SkM fibers, only in Ex-leg (representative images in Figure 2F). Representative immunofluorescence images showed an increase in LC3, and colocalization with TOM20 in Ex-leg compared to Rest-leg in both IMF and SSM mitochondria subpopulations (Figure 2G). The decrease in mitophagy proteins abundance BNIP3L/NIX and PARKIN, in addition to an elevated number of mitophagosome-like structures, suggest an increase in mitophagy, potentially with a mitochondrial ejection contribution in Ex-leg.

We confirmed our findings in a second cohort of resistance-trained volunteers who completed a two-leg resistance exercise protocol (reanalyzed data from the published study¹²). In this cohort, we found a significant increase in mitophagosome-like structures in SSM mitochondria (see protocol and results in Figure S2).

2.2 | Mice study

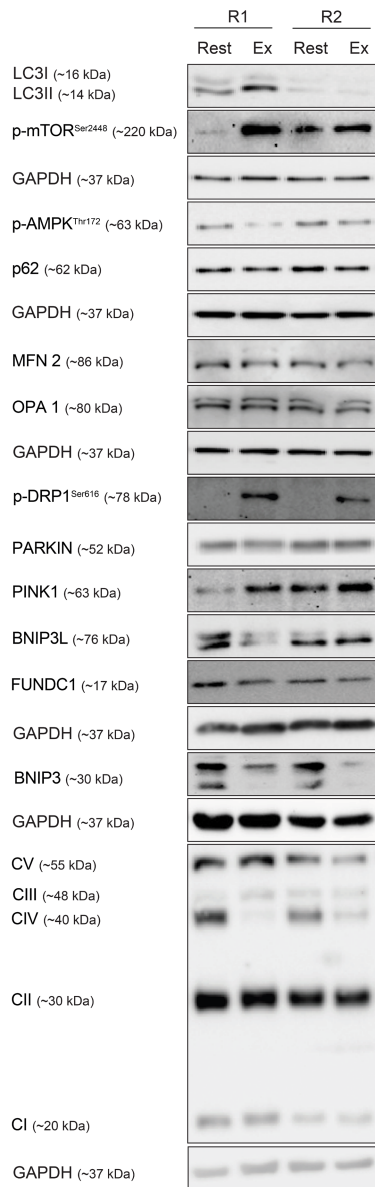
To ensure the reliability of our results, we performed the visualization of the membrane with PK-memb (Figure 3A) and mitochondria with PK-mito (Figure 3B) in live imaging isolated mice SkM fiber. Our observations revealed clear circular membrane-wrapped mitochondria (mitophagosome-like) in the subsarcolemmal region (Figure 2C; Video S1), consistent with our previous findings on fixed human muscle in TEM (Figure 2F), and immunofluorescence images (Figure 2G).

2.3 | Human study 2

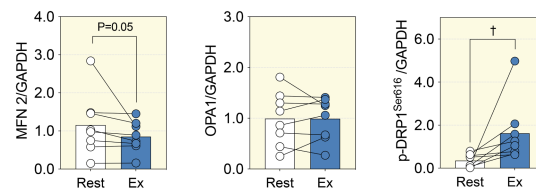
2.3.1 | Resistance exercise has no effect on the number of extracellular EVs or the mitochondrial proteins present within them

One way to eject molecules or organelles from the cell is through EVs. Blood samples were obtained from volunteers before and after a resistance exercise bout, and extracellular EVs were isolated. We did not find any differences in the number of EVs, mitochondrial OXPHOS protein abundance, or BNIP3L/NIX protein contained within EVs after acute resistance exercise (Figure S1B–H).

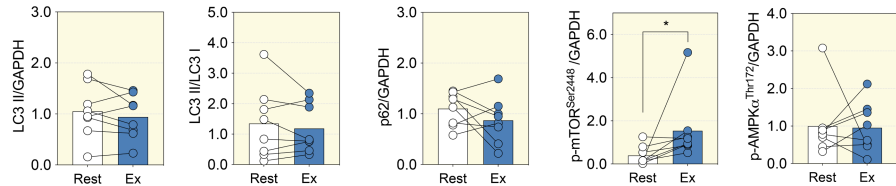
(A)



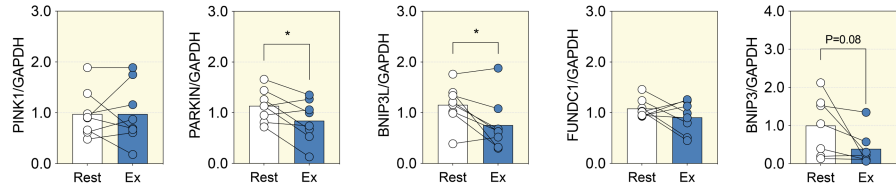
(B) Mitochondrial fusion/fission



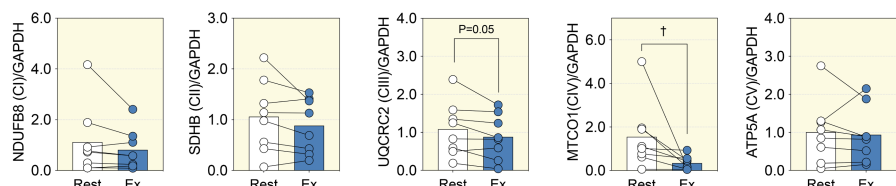
(C) Autophagy



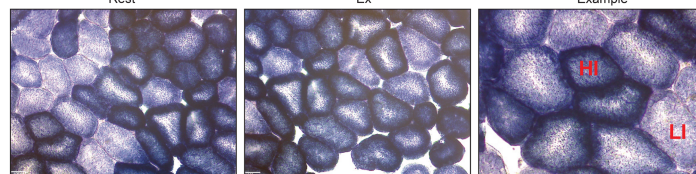
(D) Mitophagy



(E) OXPHOS



(F)



(G)

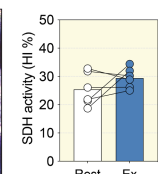


FIGURE 1 Acute resistance exercise increases p-Drp1^{Ser616} and decreases BNIP3L in human skeletal muscle. (A) Representative images of the measured proteins in the non-exercised (Rest) and exercised (Ex) leg from human study 1. Densitometry quantification of proteins for (B) mitochondrial fusion and fission, (C) autophagy, (D) mitophagy, and (E) oxidative phosphorylation (OXPHOS). (F) Representative image of succinate dehydrogenase (SDH) staining. HI, high intensity; LI, low intensity. (G) Quantification of SDH staining. Statistical significance was tested using a paired *t*-test. **p* < 0.05, †*p* < 0.01. Dots in the bar graphs represent different participants, and the data are shown as means.

2.4 | Human primary myotube study

2.4.1 | BNIP3L/NIX decreases in response to mitophagy induction in human primary myotubes

As BNIP3L/NIX and PARKIN protein abundance decreased in response to resistance exercise, we tested if, in human myotubes from healthy men, these proteins also responded to mitophagy induction. Myotubes

were incubated with the mitophagy inducer CCCP²⁷ and co-incubated with bafilomycin to inhibit lysosomal degradation.²⁸ Bafilomycin addition resulted in higher BNIP3L/NIX protein abundance in the basal condition (Figure 4A,C). BNIP3L/NIX decreased in response to CCCP, in the absence and presence of bafilomycin. The CCCP or bafilomycin did not modify neither PARKIN nor BNIP3 protein abundance (Figure 4A,C). The protein abundance of the autophagy markers LC3II and p62 increased after CCCP incubation and accumulated with

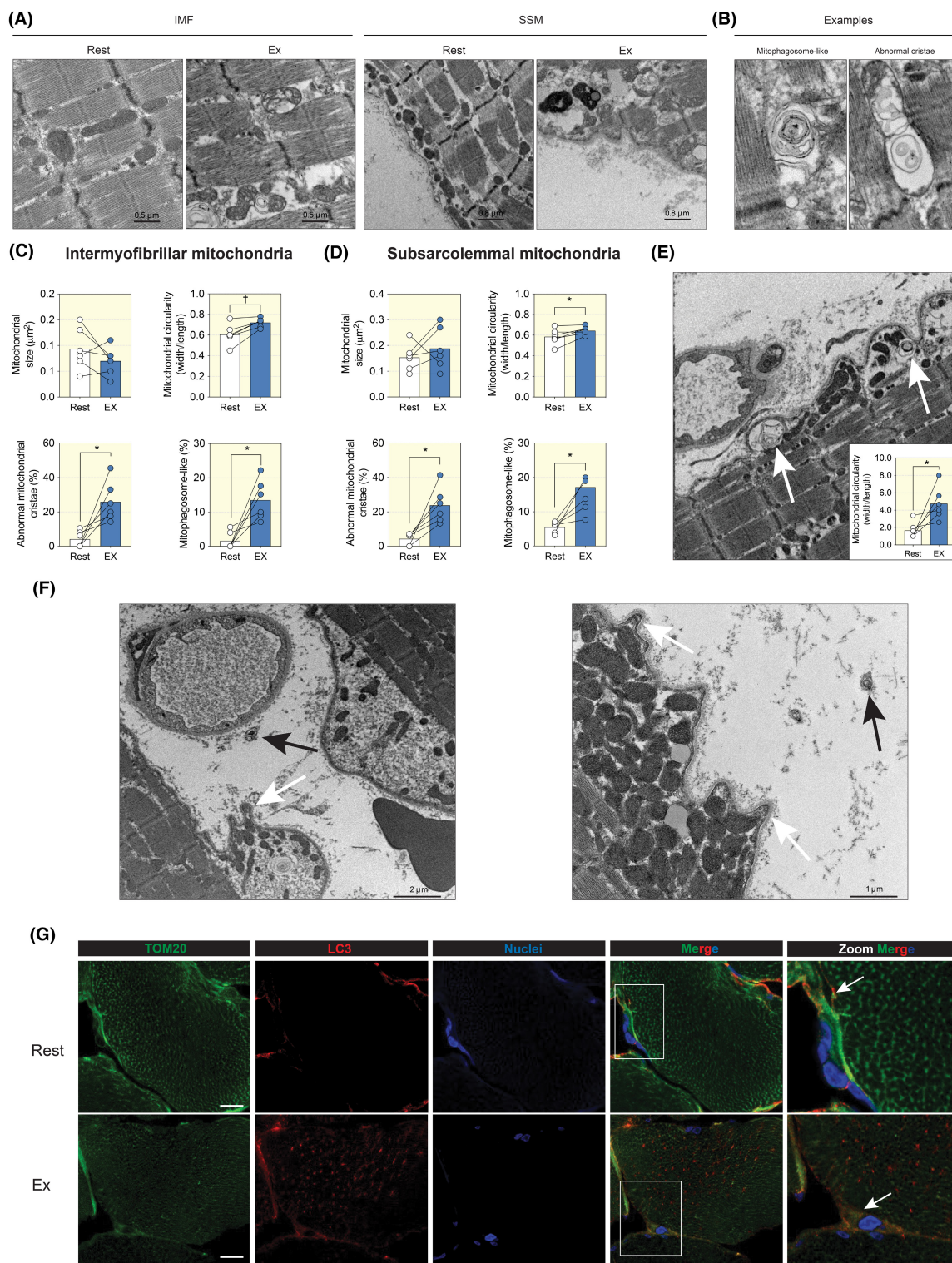


FIGURE 2 Acute resistance exercise induces mitochondrial damage, fragmentation, and increase in mitophagosome-like structures. (A) Representative transmission electron microscopy image of the intermyofibrillar (IMF) and subsarcolemmal (SSM) mitochondria. (B) Representative image of the mitophagosome-like structures and abnormal cristae. Mitochondrial morphology and mitophagosome-like structure quantification of (C) IMF and (D) SSM mitochondria. (E) Representative image and quantification of mitophagosome-like structures on cleft. (F) Example of a mitophagosome-like structure ejected outside of the myofiber in humans. White arrows indicate mitochondria on cleft and black arrows indicate mitophagosome-like structures and mitochondria outside the myofiber. (G) Representative immunofluorescence image using TOM20 (green), LC3 (red), and nuclei (blue). Scale bar = 500 μm . The white frame indicates the zoomed-in region showed on the right panel. The white arrows indicate the merged signal for LC3 and TOM20 staining on the membrane. Statistical significance was tested by a paired t -test. * $p < 0.05$, † $p < 0.01$. The dots in the bar graphs represent different participants, and the data are shown as means.

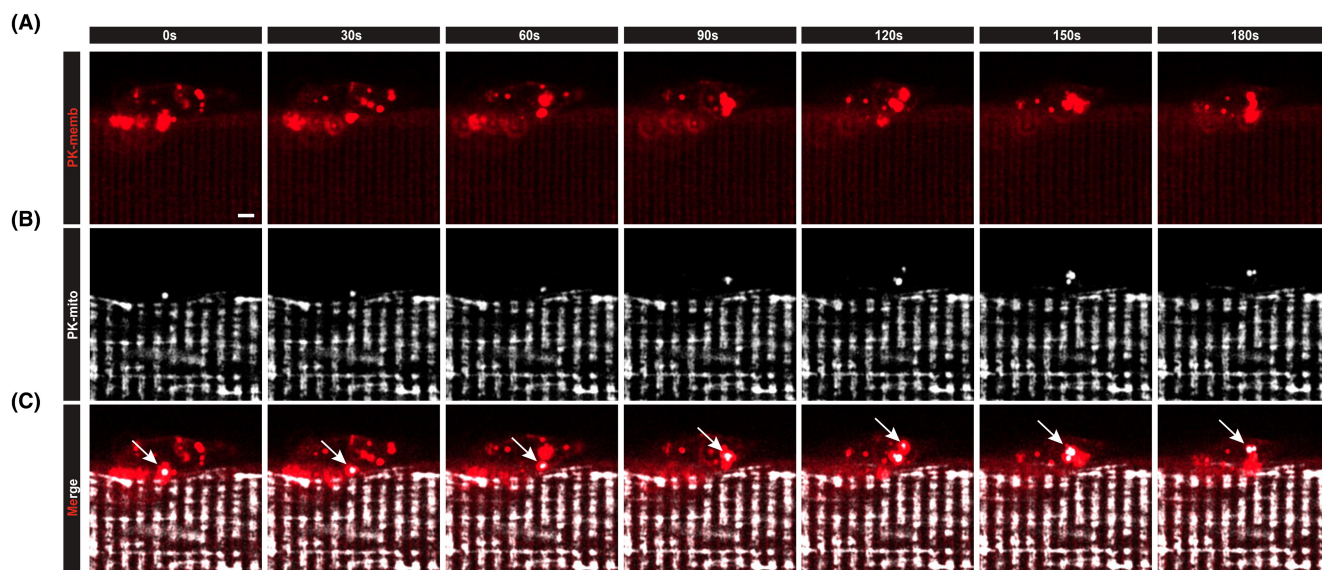


FIGURE 3 Visualization of circular membrane-wrapped mitochondria on the subsarcolemmal region in isolated skeletal muscle fiber from mice. Time course of isolated live FDB fiber dyes with (A) PK-memb, (B) PK-mito, and (C) merge. Scale bar = 2 μ m.

bafilomycin treatment in both control and CCCP conditions (Figure 4A,B). As mitochondrial biogenesis readout, we measured PGC1 α gene expression using real-time PCR. No differences were observed between the conditions (Figure 4D). These results from our in vitro mitophagy experiments, together with the resistance exercise results, suggest that muscle-released mitochondria may be guided by, or work in conjunction with, BNIP3L (see hypothesis model in Figure 4E).

2.5 | Human study 3

2.5.1 | BNIP3L/NIX is associated with mitophagosome-like structures in resting conditions in human SkM

As BNIP3L/NIX was the only protein to decrease in response to resistance exercise and CCCP-induced mitophagy, we assessed its potential as a mitophagy flux marker in human SkM. We found a negative association between BNIP3L/NIX protein abundance in human SkM and mitophagosome-like structure percentage but not number (Figure 5A–C) and we observed a tendency to an inverse correlation with cristae density (Figure 5A,D).

3 | DISCUSSION

In this study, we investigated the effects of a single resistance exercise on mitophagy in human SkM using a single-leg model. Our hypothesis was that the exercise would cause mitochondrial fission and mitophagy. The results

showed that resistance exercise increased p-DRP1^{Ser616} and reduced the protein abundance of the mitophagy markers PARKIN and BNIP3L/NIX. The exercise also increased mitochondrial fragmentation, promoting mitochondria with abnormal cristae and mitophagosome-like structures. Furthermore, we observed mitophagosome-like structures outside the SkM in the exercised leg. In human myotubes, BNIP3L/NIX protein abundance decreased due to mitophagy induction, independently of lysosomal degradation. Additionally, we observed a negative association between BNIP3L/NIX protein abundance and mitophagosome-like structures. Altogether, these results suggest that a single bout of resistance exercise can trigger mitophagy, potentially involving mitochondrial ejection in human SkM. We, therefore, propose BNIP3L/NIX as a marker of mitophagosome flux in human SkM.

Mitochondrial fission is a critical step in mitophagy, and our findings indicate that resistance exercise triggers mitochondrial damage (e.g., abnormal cristae), a fission phenotype (as evidenced by p-DRP1^{Ser616} levels and mitochondrial circularity), and an upsurge in mitophagy (evidenced by increases in mitophagosome-like structures and decreases in mitophagy-associated proteins). Similarly, Botella et al. reported reduced aspect ratios, increased circularity, and heightened signs of mitochondrial damage following a resistance exercise bout in strength athletes.¹² In contrast, another study found no significant differences in the abundance of mitochondrial fission proteins after 24 h of resistance exercise.²⁹ We postulated that resistance exercise would stimulate mitophagy, leading to the removal of damaged mitochondria in SkM. We supported this hypothesis by observing increased external mitophagosomes and diminished BNIP3L/NIX and

□ Veh
■ Bafilomycin A1

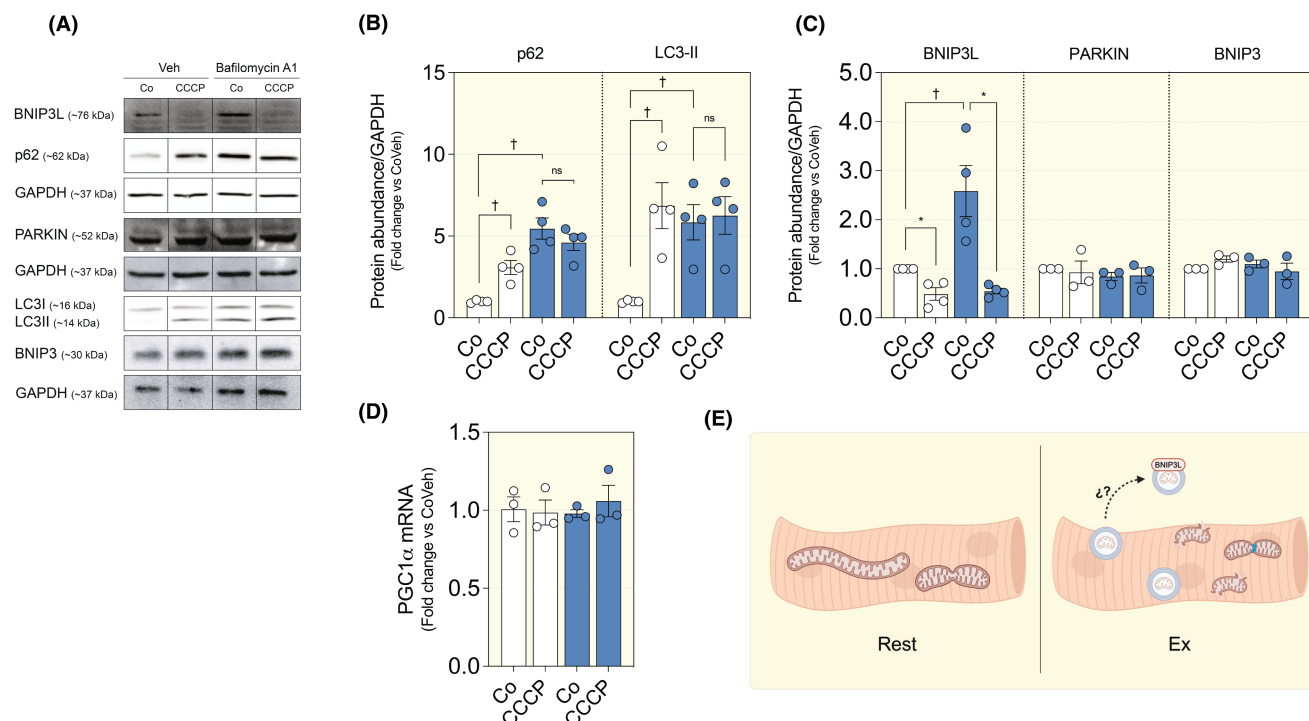


FIGURE 4 BNIP3L decreases in response to mitophagy induction and is independent of lysosomal degradation. (A) Western blot images of BNIP3L, p62, PARKIN, LC3I-II, BNIP3, and GAPDH from human myotubes stimulated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and/or bafilomycin A1. (B) p62 protein quantification and LC3-II protein ratio quantification (C) BNIP3L, PARKIN, and BNIP3 protein quantification. (D) PGC1α mRNA expression. (E) Graphical representation of the hypothesized model of mitochondria ejection from the human myofiber mediated by BNIP3L in response to resistance exercise and CCCP. The conditions shown were obtained from the same western blot gel. Statistical significance was tested by a two-way ANOVA and post-hoc analysis. * $p < 0.05$, † $p < 0.01$. $n = 3-4$. The dots in the bar graphs represent independent experiments, and the data are shown as mean $\pm$ SEM.

PARKIN protein abundance. Notably, while the expulsion of mitochondria from cells has been explored in other models,³⁰⁻³² our study marks the first observation of this phenomenon in human SkM following exercise. The fact that resistance exercise acutely dampens phosphorylation sites involved in mitophagy (OPTN and p62³³) may suggest that an increased activation of alternative mitophagy pathways is needed to ensure the removal of dysfunctional organelles. Besides, we did not find differences between mitochondria EVs cargo before and after resistance exercise in plasma. We hypothesize that exercise-induced mitochondrial ejection could be directed to tissue-resident macrophages, as previously observed in cardiac muscle²³ and as we observed in the TEM images (Figure 2E,F).

Our in vitro human myotube experiments showed that BNIP3L/NIX was the sole mitophagy marker that decreased in response to mitophagy induction, independently of lysosomal degradation. This did not affect PGC1α mRNA expression. However, since mitophagy may regulate mitochondrial biogenesis,³⁴ we cannot dismiss the possibility of mitochondrial biogenesis regulation in

human SkM in response to resistance exercise, through a PGC1α-independent pathway or through posttranslational modifications of PGC-1α. These findings led us to propose that BNIP3L/NIX could play a role in ejecting damaged mitochondria from human SkM after mitochondrial injury due to resistance exercise, since a recent report on cardiac muscle has shown that damaged mitochondria are secreted in extracellular vesicles when lysosomal degradation is impaired.²³ In the context of resistance exercise, with a significant level of mitochondrial damage, possibly surpassing the lysosomal degradation capacity of SkM, there might be an increased need for expelling these damaged mitochondria to other cell types possessing greater lysosomal capacity. In this scenario, BNIP3L/NIX could be a marker for these ejected mitochondria.

BNIP3L/NIX is a mitophagy receptor from the BCL-2 family, regulating apoptosis, necrosis, autophagy, and mitophagy.^{35,36} Its role in maintaining mitochondrial health in SkM has been documented in rodent and in vitro models^{21,37}; however, its role in human SkM remains unexplored. The lack of changes in PARKIN protein abundance

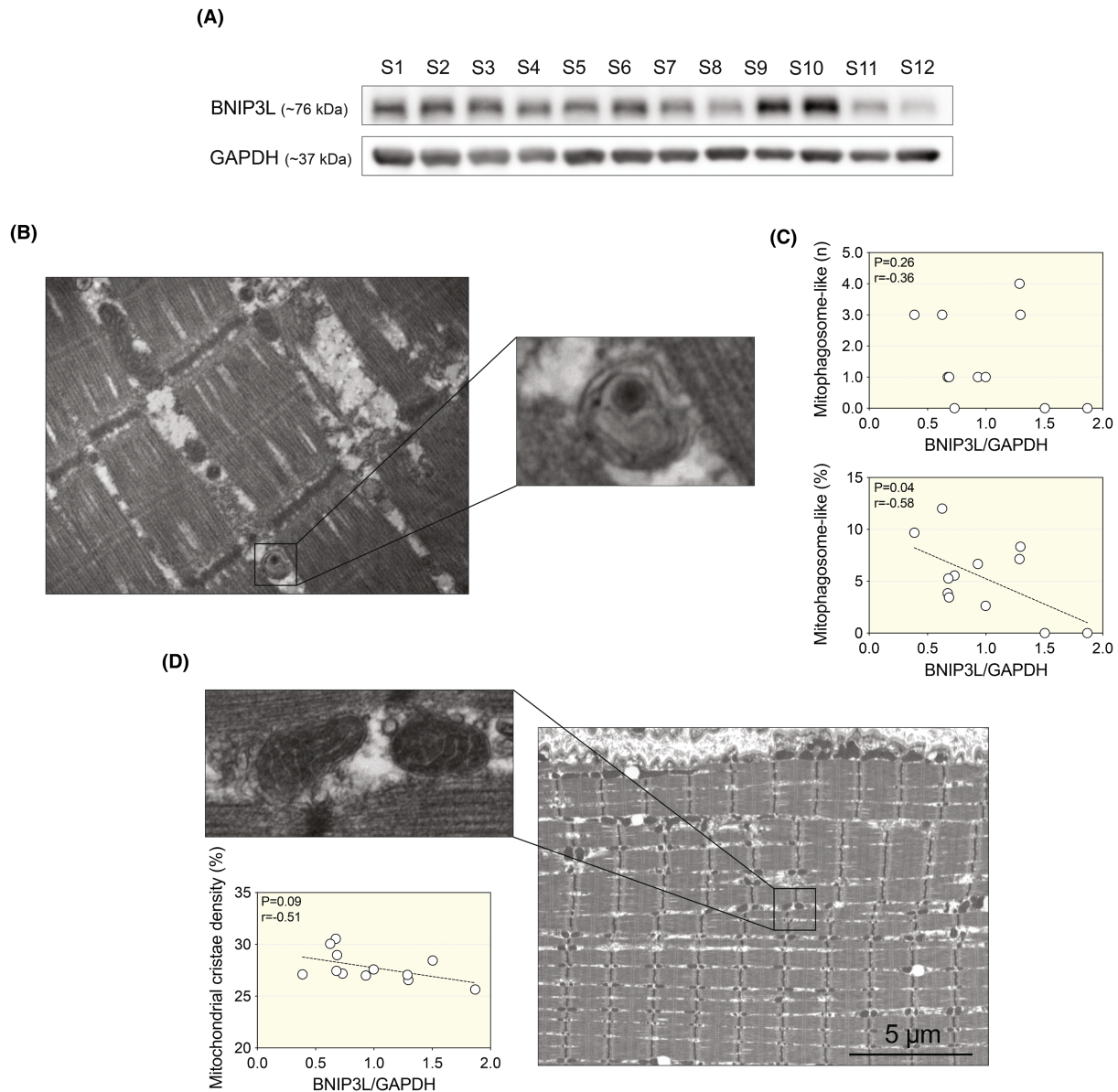


FIGURE 5 BNIP3L correlates with the percentage of mitophagosome-like structures in human skeletal muscle. (A) BNIP3L and GAPDH protein expression in the 12 participants of human study 3. (B) Representative image of a mitophagosome-like structure. (C) Correlation between mitophagosome-like structure number/percentage and BNIP3L expression. (D) Representative image of mitochondria cristae and its correlation with BNIP3L expression. Correlations were tested using the Pearson test. The dots in the bar graphs represent different participants.

in response to CCCP was an unexpected result; however, the main pathway of mitophagy in human SkM has not yet been clearly established and moreover mitophagy has been shown to be induced independently of PINK1-Parkin on SkM during exercise.³⁸

Given BNIP3L/NIX responsiveness to resistance exercise and CCCP treatment, we tested the possible role of BNIP3L/NIX as a mitophagy flux marker in human SkM. Our findings suggest that BNIP3L/NIX accumulation may be indicative of the extent of mitochondrial damage, as there was a negative association between BNIP3L/NIX abundance and both the percentage of

mitophagosome-like structures and mitochondrial cristae density in SkM. We also found that only BNIP3L/NIX, but not PINK1, PARKIN, or BNIP3, was associated with mitophagosome-like structures in resting conditions (data not shown). This suggests that elevated levels of PINK1, PARKIN, or BNIP3 at rest could indicate an increased capacity for the mitophagy process. In contrast, increases in BNIP3L/NIX levels at rest could indicate mitochondrial damage accumulation and a decrease in mitophagy flux. Further experiments involving the inhibition of auto/mitophagy at various stages are needed to validate this hypothesis.

4 | MATERIALS AND METHODS

4.1 | Human study 1

4.1.1 | Volunteers characteristics and one-leg resistance exercise protocol

Eight non-diabetics, non-smoking men without any history of cardiovascular, respiratory, or thyroid disease were included (see characteristics of the volunteers in Table 1). All subjects signed a written informed consent approved by the Institutional Review Board of the Universidad Finis Terrae (Comité ético científico Universidad Finis Terrae, Santiago, Chile, IDP:22-020), and the study adhered to the Declaration of Helsinki.

First appointment

After an 8-h fasting period and a 48-h abstinence from caffeine and alcohol consumption, subjects arrived at the laboratory with minimal physical activity. Anthropometric measurements were taken, including weight and height. Additionally, a one-repetition maximum (1RM) test (see details later) was performed on the dominant leg.

Second appointment

One week after the determination of the 1RM, the subjects arrived at the laboratory in the same condition as in the first appointment and performed an acute resistance exercise with the dominant leg. One hour after the exercise, a biopsy was taken from both legs (Figure 6).

4.1.2 | One repetition maximum determination

The determination of the 1RM was performed on a leg press machine. The warm-up consisted of 8–10 repetitions at 50% of the estimated 1RM, followed by 5 repetitions at

70%, 3 repetitions at 80%, and 1 repetition at 90% of the estimated 1RM. Thereafter, the load was increased by 2 kg increments until the subject could perform only one repetition with the highest possible load. Subjects rested for 3–5 min between stages. The 1RM was recorded as the final load successfully lifted.

4.1.3 | One-leg resistance exercise and SkM biopsies

After a warm-up on the leg press machine (10 repetitions with 22 kg), the exercise session consisted of 10 series of 10 repetitions at 70% of 1RM with 2 min rest between series with the dominant leg. One hour after exercise, a biopsy was taken from the vastus lateralis SkM of the non-exercised (Rest-leg) and exercised leg (Ex-leg). The biopsies were split into three pieces: (1) immediately frozen in liquid nitrogen and stored at -80°C for western blotting; (2) fixed in 2.5% glutaraldehyde and stored at room temperature for TEM analyses; (3) excess blood and fat were removed using 1x PBS, then the SkM fibers were transversely oriented, frozen in isopentane, and stored at -80°C for immunofluorescence microscopy.

4.1.4 | Western blot

SkM biopsies were homogenized using an electric homogenizer in a buffer containing: 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 2 mM EDTA, 20 mM NaF, 1 mM $\text{Na}_2\text{P}_2\text{O}_7$, 10% glycerol, 150 mM NaCl, 10 mM Na_3VO_4 , 1 mM PMSF, and a protease inhibitor cocktail (complete mini; Roche Applied Science). Proteins were separated by SDS-PAGE and transferred to PVDF membranes, as previously described.³⁹ The following antibodies (dilutions) were used: MFN2 (1:1000, ab56889, Abcam), OPA1 (1:1000, 612 606, BD Biosciences), total OXPHOS cocktail (1:1000, ab110413, Abcam), LC3 (1:1000, 4108, Cell Signaling Technology), p-mTOR^{Ser2448} (1:1000, 2971, Cell Signaling Technology), p-AMPK^{Thr172} (1:1000, 2531, Cell Signaling Technology), p62 (1:2000, H00008878-M01, Abnova), p-DRP1^{Ser616} (1:1000, SC-101270, Santa Cruz Biotechnology), PINK1 (1:1000, NB.BC100-494, Novus Biological), PARKIN (1:1000, SC-32282, Santa Cruz Biotechnology), BNIP3L/NIX (1:1000, 12 396, Cell Signaling Technology), BNIP3 (1:1000, ab10433, Abcam), and FUNDC1 (1:1000, ab224722, Abcam). GAPDH (1:10000, 2118, Cell Signaling Technology) was used as a loading control in all gels. Protein bands were visualized on a film (WESTAR SUPERNOVA detection kit, Cyanagen, Bologna, Italy), scanned, and quantified by densitometry using Image

TABLE 1 Participants' characteristics of human study 1.

<i>n</i> = 8	Mean (SD)
Age (years)	31.50 (4.23)
Height (m)	1.72 (0.05)
Weight (kg)	78.45 (6.29)
IMC (kg/m^2)	26.34 (1.66)
Glucose (mg/dL)	88.50 (3.89)
Insulin (UI/mL)	7.53 (2.08)
HOMA—IR index	1.66 (0.53)
$\text{VO}_{2\text{max}}$ (L/min)	3.13 (0.42)
$\text{VO}_{2\text{max}}$ (mL/kg/min)	40.09 (5.81)
1RM dominant leg (kg)	111.67 (25.08)

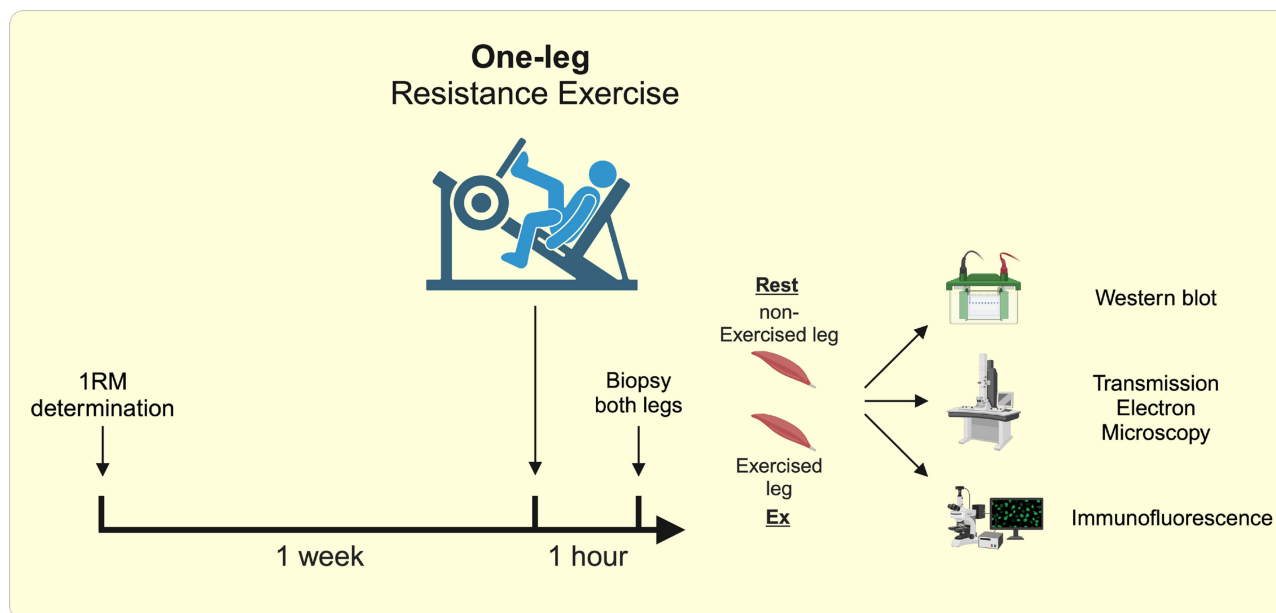


FIGURE 6 Schematic representation of human study 1.

Lab Software Version 6.0 (Bio-Rad). For each evaluated protein, the densitometry of the Ex and Rest-leg was normalized to the average of the Rest-leg.

4.1.5 | Succinate dehydrogenase stain

To evaluate markers of mitochondrial oxidative capacity, SkM sections of 10 μm thickness were made using a cryostat (Leica Biosystems, IL, USA) at -20°C . Enzymatic activity of SDH was assayed by placing the slides in succinate dehydrogenase (SDH) incubating solution, containing sodium succinate as a substrate and nitro-blue tetrazolium (NBT) for visualization of the reaction for 1 h at 37°C .⁴⁰ The activity was quantified in approximately 70 fibers per volunteer using Fiji/ImageJ software, categorizing the fibers as high intensity (HI) or low intensity (LI). The results were represented as the percentage of high-intensity fibers from the total fibers.

4.1.6 | TEM samples preparation and image analysis

Biopsies were fixed in a glutaraldehyde solution (2.5%, room temperature, 2 h). Fixed SkM was dissected into bundles of fibers, washed four times with 0.1 M sodium cacodylate buffer, and stained with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h. Samples were washed with water, stained with 1% uranyl acetate for 2 h, dehydrated on acetone dilution series, and embedded in Epon resin. Finally, 80-nm sections were cut,

mounted on grids, and examined using a TEM (Talos F200C G2; Microscopy Facility, Pontificia Universidad Católica de Chile). For mitochondrial morphology, a mask was drawn over the outer membrane of the mitochondria at $\times 15000$ magnification, as described.^{39,41} The mitophagosomes-like were identified as previously described^{42,43} and are presented as number (n) and percentage with respect to total mitochondria number (%). TEM images were analyzed on fiber type 1 in longitudinal sections using Fiji/ImageJ software. Mitophagosome-like structure was identified as previously reported.⁴⁴ These evaluations were carried out in conditions where the evaluators were unaware of the subject's identity (blinded conditions).

4.1.7 | Fluorescence microscopy

SkM sections of 10 μm thickness were made using a cryostat (Leica Biosystems, IL, USA) at -20°C . Fixed SkM sections were blocked with 3% bovine serum albumin (BSA, Winkler, Chile) in phosphate-buffered saline (PBS) and permeabilized with 0.25% Triton X-100 for 1 h at room temperature. Then, SkM sections were incubated overnight at 4°C with the primary antibody against TOM20 (sc-136 211, Santa Cruz) and LC3 (4108, Cell Signaling Technology) prepared in 0.05% Triton X-100 and 3% BSA in PBS, followed by conjugation with its secondary antibody (Alexa Fluor, Life Technologies, Carlsbad, CA, USA), for 1 h at room temperature. Before the mounting medium, nuclei were stained with DAPI (1:10000, 12 min) at room temperature. Confocal

fluorescence microscopy assessments were performed using an LSM 880 Zeiss inverted confocal microscope with Airyscan detection (Unidad de Microscopía Avanzada UC [UMA UC]). Contrast and/or brightness adjustment and cropping of final images were done using ImageJ, with identical settings applied to all images from the same experiment.

4.2 | Mice study

4.2.1 | Fiber isolation

Mice experiments were approved by the Danish Animal Experimental Inspectorate (#2015-15-0201-00477) and complied with the “European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes.” C57BL/6 mice were sacrificed by cervical dislocation, and the flexor digitorum brevis (FDB) muscle was removed under a dissection microscope. Isolated live muscle fibers were obtained by enzymatic digestion of the whole muscle in α -MEM (22571-020, Gibco) containing 2.0 mg/mL collagenase type 1 from *Clostridium histolyticum* (C0130, Sigma-Aldrich) for 90–120 min at 37°C on a rotator. After collagenase treatment, individual muscle fibers were obtained by mechanical dissociation using fire-polished Pasteur pipettes. Isolated fibers were seeded overnight in ECM Gel-coated (E1270, Merck) 35 $\times$ 14 mm glass-bottom microwell dishes (P35G-1.5-14-C, MatTek Corporation) containing α -MEM supplemented with 10% fetal bovine serum.

4.2.2 | Fluorescent dyes

To visualize mitochondria and the plasma membrane in living cells, we employed the PK-Mito dye⁴⁵ and PK-memb dye⁴⁶ following the manufacturer's instructions (Spirochrome). In short, dyes were incubated for 45 min (mito) and 15 min (PK memb) in their regular maintenance media, then washed three times with PBS before live imaging.

4.2.3 | Live imaging procedures

Images were collected at intervals of 3 s using a 63 $\times$ 1.4 NA oil immersion objective on an LSM 980 confocal microscope (Zeiss) driven by Zeiss Zen Blue3. Adult muscle fibers were imaged in 35 mm glass-bottom microwell dishes in Krebs Ringer buffer (145 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES) while kept at 95% O₂, 5% CO₂, and 37°C using a heat stage.

4.3 | Human study 2

4.3.1 | Volunteers, two-leg resistance exercise protocol, and extracellular vesicle purification

Three subjects evaluated for the one-leg resistance exercise protocol (*human study 1*) were recruited again 4 weeks after their participation in the *human study 1*. The 1RM evaluation, as well as the resistance exercise protocol, remained the same as in *Human Study 1*. The only modification was that the resistance exercise was performed with both legs in *human study 2*, while in *human study 1* it was performed with one leg. A blood sample was taken 1 h before and 1 h after the resistance exercise to isolate extracellular vesicles (Figure S1A).

4.3.2 | Extracellular vesicle (EVs) purification from human plasma

Extracellular vesicles were purified using a modified protocol previously described.⁴⁷ Blood samples were obtained in EDTA-coated tubes, maintained at room temperature for 30 min, and centrifuged at 1200g for 10 min at 4°C to separate plasma. Then, the plasma was centrifuged again at 3000g for 15 min at 4°C. The supernatant (platelet-poor plasma) was transferred to a new tube and stored at 80°C until use. The platelet-poor plasma (~850 μ L) was thawed on ice and diluted with an equal volume of sterile 1X PBS. Then, the samples were centrifuged at 2000g for 30 min at 4°C. The supernatant (~1500 μ L) was transferred to new tube, diluted with sterile 1X PBS to complete volume in a 4-mL ultracentrifuge tube (Beckman Coulter, cat. 344062), and centrifuged at 12000g for 45 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 110000g for 2 h and 10 min at 4°C (Sorvall WX80, Thermo Scientific). The pellet obtained was resuspended in 4 mL of sterile 1X PBS and, centrifuged at 110000g for 2 h and 10 min at 4°C. Finally, the supernatant was discarded, and the pellet was resuspended in 100 μ L of sterile 1X PBS, and stored at -80°C. The concentration of EVs of each sample were determined by nanotracking analysis (Malvern Nanosight NS300).

4.3.3 | Western blot

EVs were homogenized in a RIPA buffer with a protease inhibitor cocktail (complete mini; Roche Applied Science). A volume equivalent to 1.2 $\times$ 10⁹ EVs per subject pre-and post-exercise was then loaded and proteins from EVs were separated by SDS-PAGE and transferred to PVDF membranes, as previously described.³⁹ The

following antibodies (dilutions) were used: CD81 (1:500, SC-7637, Santa Cruz), Alix (3A9) (1:500, SC-53538, Santa Cruz), and TSG101 (1:1000, ab30871, Abcam). Protein bands were visualized on a film (WESTAR SUPERNOVA detection kit, Cyanagen, Bologna, Italy), scanned, and quantified by densitometry using Image Lab Software Version 6.0 (Bio-Rad).

4.4 | Human primary myotubes study

Myoblasts from three healthy humans were evenly pooled and differentiated to obtain myotubes as described previously.⁴⁸ Myotubes were treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μ M) and bafilomycin (100 nM) for 24 h. Dimethyl sulfoxide (DMSO) was used as a vehicle in the experiment. Then cells were lysed with T-per buffer (78 510, Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (complete mini; Roche Applied Science).

4.4.1 | Western blot

Cells were lysed in RIPA buffer, and 30–40 μ g of denatured proteins from each sample were resolved in 10% SDS-PAGE. Gels were transferred to nitrocellulose membranes and incubated with 5% BSA (BM-0150, Winkler)-tris buffered saline-0.1% Tween-20 (TBS-T) to block nonspecific binding. Membranes were incubated with the following primary antibodies: BNIP3L/NIX (1:1000, 12 396, Cell Signaling Technology), p62 (1:2000, H00008878-M01, Abnova), PARKIN (1:1000, SC-32282, Santa Cruz Biotechnology), LC3 (1:1000, 4108, Cell Signaling Technology), BNIP3 (1:1000, ab10433, Abcam), GAPDH (1:10000, 2118, Cell Signaling Technology) was used as a loading control in all gels. Protein bands were visualized on a film (WESTAR SUPERNOVA detection kit, Cyanagen, Bologna, Italy), scanned, and quantified by densitometry using Image Lab Software Version 6.0 (Bio-Rad). For each evaluated protein, the densitometry was normalized to the average of the control condition. Four independent experiments were performed.

4.4.2 | Real time PCR (RT-PCR)

For analysis of PGC1 α gene expression in human primary myotubes, RNA was extracted using the E.Z.N.A.R Total RNA Kit (OMEGA Bio-Tek, Norcross, GA, USA) according to the manufacturer's indications. cDNA was synthesized using iScriptTM cDNA kit (Bio-Rad) from 1 μ g of total RNA. Quantitative PCR reactions

were carried out on a Step One System Real Time PCR (Applied Biosystems) using Fast SYBR GreenMaster Mix (4385370, Life Technologies). Specific forward and reverse primers sequences used to evaluate gene expression PGC1 α are: Forward: AGCCTCTTTGCCAGATCTT. Reverse: GGCAATCCGTCTTCATCCAC. GAPDH: Forward: ACATCGCTCAGACACCATG. Reverse: TGTA GTTGAGGTCAATGAAGGG. GAPDH was used as a housekeeping gene. The $\Delta\Delta$ CT method was used for relative quantification analysis.

4.5 | Human study 3

We conducted a secondary analysis using partial data from our previous study.³⁹ In this analysis, we assessed the number and proportion of mitophagosome-like structures and mitochondrial cristae density in TEM images of 12 healthy individuals at rest. These evaluations were carried out in conditions where the evaluators were unaware of the subject's identity (blinded conditions). Further details about the materials and methods can be found in our previous study.² The data obtained were linked to BNIP3L/NIX protein abundance, which was evaluated through western blot, as described in point 2.1.4.

4.6 | Statistics

Data are presented as means and standard deviation (SD). Paired *t*-test was performed to analyze differences between the Rest-leg and Ex-leg, and pre- and post-exercise plasma EVs. Correlations between study variables were assessed with the Pearson test. For myotubes experiments, data are presented as means and standard error of the mean (SEM). Two-way ANOVA was performed (after logarithmic transformation). For all studies, $p < 0.05$ was considered significant, and $p \geq 0.05$ and $p < 0.1$ was considered a trend. Statistical analyses were performed using Prism 9 Analyses (GraphPad Software, La Jolla, CA).

5 | CONCLUSION

In conclusion, our findings demonstrate that resistance exercise triggers mitochondrial fission and mitophagy in human SkM. We propose a novel mechanism for mitochondrial clearance, operating independently of lysosomal degradation and mediated by BNIP3L/NIX in SkM. However, further investigations, including mitochondrial tracking to validate their expulsion from SkM or BNIP3L/NIX deletion while performing at resistance exercise, are

imperative to comprehensively grasp the role of BNIP3L/NIX in mitochondrial response and adaptation to resistance exercise.

AUTHOR CONTRIBUTIONS

Francisco Díaz-Castro: Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; data curation; supervision; resources. **Mauro Tuñón-Suárez:** Investigation; validation; methodology; formal analysis. **Patricia Rivera:** Investigation; methodology; validation; formal analysis. **Javier Botella:** Investigation; methodology; validation; writing – review and editing; formal analysis; data curation. **Jorge Cancino:** Investigation; methodology; validation. **Ana María Figueroa:** Investigation; methodology; validation. **Juan Gutiérrez:** Investigation; methodology; validation. **Claudette Cantin:** Investigation; methodology; validation. **Louise Deldicque:** Investigation; writing – review and editing; validation; methodology. **Hermann Zbinden-Foncea:** Validation; investigation; writing – review and editing. **Joachim Nielsen:** Investigation; writing – review and editing; validation; methodology; visualization; project administration; supervision. **Carlos Henríquez-Olguín:** Investigation; methodology; visualization; validation. **Eugenia Morselli:** Investigation; methodology; validation; writing – review and editing; project administration; supervision; resources; funding acquisition; visualization. **Mauricio Castro-Sepúlveda:** Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; visualization; validation; methodology; software; formal analysis; project administration; resources; supervision; data curation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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SUPPORTING INFORMATION

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